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14. ABSTRACT
Schwannomas are benign peripheral nerve tumors that occur in individuals with neurofibromatosis 2 and schwannomatosis. Although schwannomas occur due to mutations in the neurofibromatosis 2 gene, which encodes the merlin tumor suppressor protein, recent studies indicate that schwannoma growth may depend in part on signaling by the erbB2 receptor tyrosine kinase. We previously found that erbB2 signaling depended on interactions between erbB2 and the CD44 transmembrane glycoprotein. CD44 is the receptor for hyaluronan, a glycosaminoglycan found in most extracellular matrices. Here, we found that schwannomas contain 2-3 fold higher levels of a high molecular weight form of hyaluronan compared to normal human peripheral nerve tissue. This elevated hyaluornan is due in part to increased transcription of the hyaluronan synthase gene, HAS2. Elevated hyaluronan correlated with increased phosphorylated (e.g. active) erbB2. These data support the hypothesis that hyaluronan may contribute to schwannoma growth.

15. SUBJECT TERMS
schwannoma, hyaluronan, erbB2, CD44

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Introduction

Schwannomas are benign peripheral nerve sheath tumors comprised of aberrant Schwann cells, and are the hallmark of the the tumor pre-disposition syndromes neurofibromatosis 2 (NF2) and schwannomatosis. We and others found that the glycosaminoglycan hyaluronan (HA) is present in the extracelluar matrix of schwannomas from patients with (NF2). HA, which can reach sizes >10^6 Da, is synthesized by 3 different transcriptionally regulated HA synthases. Different sizes of HA have diverse activities including regulating cell proliferation and differentiation. Cells respond to HA through one of several transmembrane HA receptors including CD44, layilin, the receptor for HA-mediated motility (RHAMM), and Toll-like receptors 2 and 4. Among these receptors, CD44 interacts with the *NF2* gene product merlin, a tumor suppressor protein[1]. A high molecular weight form of HA can induce merlin dephosphorylation and activation via CD44[1]. Interestingly, aberrant CD44 splice variants that have enhanced HA affinity are expressed by schwannoma cells that lack merlin *in situ*, suggesting that HA signaling may be amplified within schwannomas. How HA influences schwannoma growth has not been investigated.

We previously found that CD44 potentiates the heterodimerization and activation of the erbB2 and erbB3 receptor tyrosine kinases in Schwann cells[2]. In normal Schwann cells, erbB2 activation promotes Schwann cell proliferation and differentiation while chronic erbB2 activation results in Schwann cell tumorigenesis[3]. Recent data suggest that erbB2 is aberrantly activated in schwannomas from NF2 patients and that this activation may result from an autocrine loop involving neuregulins, which are erbB3 ligands [e.g. ref. 4]. However, erbB2 can also be activated by HA through both CD44-dependent and independent mechanisms[5]. **Here, we postulate that HA promotes cell proliferation in schwannomas through an erbB2- and CD44-dependent mechanism.**

Our original specific aims were to: **(1)** Determine the quality of HA in human schwannomas and how it accumulates; **(2)** Determine the profile of HA receptors expressed by schwannomas and which receptors are required for HA signaling by schwannoma cells; and **(3)** Test if HA promotes proliferation in schwannoma cells and Schwann cells that lack merlin in an erbB2-dependent manner. **Our long-term goal is to determine whether inhibiting HA synthesis, degrading HA, or blocking HA signaling might be efficacious strategies to inhibit or at least slow schwannoma growth in patients with NF2.**

Body

Our first task was to analyze the levels of HA and the quality of HA found in Schwannomas from NF2 patients as compared with normal human peripheral nerve tissue. We examined schwannoma tissues from 8 NF2 patients (including multiple tumors from the same patient) and 6 samples of normal peripheral nerve. We unexpectedly spent considerable time optimizing these protocols. We were able to extract HA and protein from 6 of the schwannomas and 3 of the normal nerves. Analysis by size exclusion chromatography indicated that the

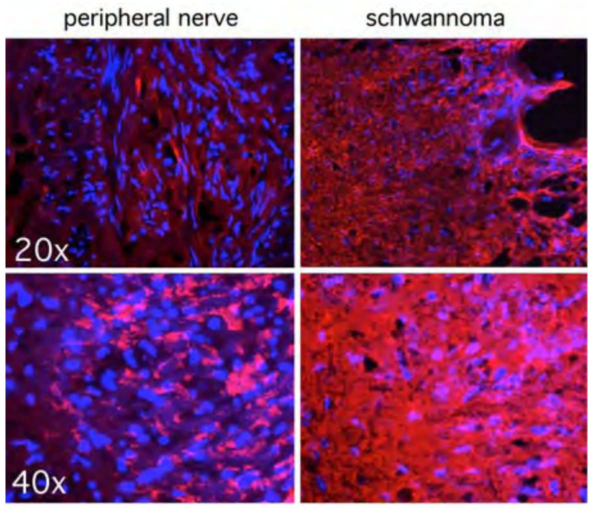

Figure 1: HA accumulates in schwannomas. Tissues were labeled with a biotinylated HA-binding protein (red) and stained with DAPI (blue) to identify cell nuclei. Peripheral nerve samples are on the left, schwannomas are on the right.

majority of the HA in both peripheral nerves and schwannomas was 1-2 x 10^6 Da, although there were also abundant lower molecular weight HA species in the schwannomas, suggesting that there may be hyaluronidase activity in these tumors. We quantified the levels of HA in these tissues using an ELISA-based assay (commercially available from Echelon Biosciences Inc.) and found that schwannomas contained 3-4 times more HA than normal peripheral nerve samples. Consistent with this finding, HA was diffusely distributed throughout normal peripheral nerve but was expressed at high density throughout all of the schwannomas as assessed by histochemistry utilizing a biotinylated HA-binding protein (**Fig. 1**). Together, these data indicate that high molecular weight forms of HA as well as breakdown products of HA accumulate in schwannomas.

We were able to obtain good quality RNA from 3 of the schwannomas and 2 of the normal peripheral nerve samples. We tested the possibility that HA accumulation in schwannomas is linked to transcriptional upregulation of HA synthases (HASs) by performing real-time PCR assays using primers against human HAS1, HAS2, and HAS3. There was 1.6-2.8 times more HAS2 RNA in the schwannomas as compared to normal nerve, while we could not detect any differences in HAS1. HAS3 was not amplified in any of the samples that we tested. These data indicate that HA accumulation is at least in part linked to increased HAS2 transcription.

Although we were not able to grow human schwannoma cells for in vitro analysis during the course of this study, we did analyze sections of schwannomas and peripheral nerves for the expression of different HA transmembrane receptors. Consistent with our previous findings[6], schwannoma expressed significantly higher levels of CD44 as compared to normal peripheral nerve as assessed by immunocytochemistry. We could not detect RHAMM in any of our samples (data not shown). Consistent with previous reports indicating that Schwann cells express TLR2[7], we also detected TLR2 in both normal peripheral nerve Schwann cells and in schwannomas, and we are currently optimizing conditions to compare TLR2 levels in these tissues by Western blotting.

Because of the unexpected technical problems we had with the biochemical characterization of HA in the archived tissue samples, we only

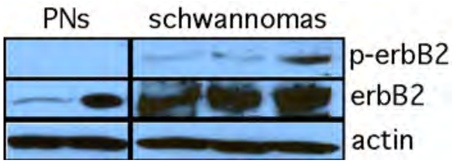

Figure 2: Schwannomas have increased erbB2 phosphorylation. PNs = peripheral nerves; p-erbB2 = phosphorylated erbB2; erbB2 = pan erbB2.

recently began analyzing the relationship between HA and erbB2 activation in schwannomas. Our first goal was to confirm that erbB2 phosphorylation is constitutively elevated in schwannomas. As shown in Fig. 2, erbB2 phosphorylation and total erbB2 expression were significantly elevated in schwannomas as compared with normal peripheral nerve tissue. Preliminary immunohistochemical analyses confirm that erbB2 phosphorylation is highest in areas within schwannomas where HA accumulates (data not shown). We are now testing how HA influences erbB2 phosphorylation in Schwann cells in vitro and comparing this activation in schwannoma cells. We hope to complete these studies in the next several months.

Key Research Accomplishments

- discovered that large amounts of HA accumulate within schwannomas from patients with NF2
- discovered that HA accumulation is linked to increased transcription of HAS2
- found that erbB2 is constitutively active within schwannomas wherever HA accumulates

Reportable Outcomes

None at this time. We are continuing our studies and aim to submit a manuscript describing our results within a year.

Conclusions

So far, we have shown that HA and HA breakdown products accumulate in schwannomas and that this accumulation correlates with elevated erbB2 phosphorylation. We are now focusing on how HA influences Schwann cell proliferation in the presence and absence of merlin. The finding that schwannoma cells may express TLR2 is interesting in light of the apparent accumulation of HA breakdown products within the tumors. TLR2 can influence Schwann cell survival[7] and is activated in response to HA breakdown products. It would therefore be interesting to examine whether Schwann cell survival is altered by HA breakdown products through a TLR2-dependent pathway. We plan to pursue this question in future studies.

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Appendices
None.